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Report

The CRYPTOCHROME Photoreceptor Gates PDF Neuropeptide Signaling to Set Circadian Network Hierarchy in *Drosophila*

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Summary

Circadian clocks in the brain are organized as coupled oscillators that integrate seasonal cues such as light and temperature to time daily behaviors. In *Drosophila*, the PIGMENT DISPERSING FACTOR (PDF) neuropeptide-expressing morning (M) and non-PDF evening (E) cells are coupled cell groups important for morning and evening behavior, respectively. Depending on day length, either M cells (short days) or E cells (long days) dictate both the morning and the evening phase, a phenomenon that we term network hierarchy. To examine the role of PDF in light-dark conditions, we examined flies lacking both the PDF receptor (PDFR) and the circadian photoreceptor CRYPTOCHROME (CRY). We found that subsets of E cells exhibit molecular oscillations antiphase to those of wild-type flies, single *cry* mutants, or single *Pdfr* mutants, demonstrating a potent role for PDF in light-mediated entrainment, specifically in the absence of CRY. Moreover, we find that the evening behavioral phase is more strongly reset by PDF(+) M cells in the absence of CRY. On the basis of our findings, we propose that CRY can gate PDF signaling to determine behavioral phase and network hierarchy.

Results

Flies Deficient for PDF Signaling and CRY Exhibit Morning Behavior But No Evening Behavior in Light-Dark Cycles

Fruit flies exhibit bimodal rest-activity profiles under light-dark (LD) conditions with circadian-clock-regulated activity peaks that anticipate dawn and dusk (termed morning- and evening-anticipatory behavior). To examine the role for PDF signaling in light-mediated entrainment, we examined locomotor behavior in flies mutant for both *Pdfr* and *cry* ($Pdfr^{han5304}$;cry^{b}) under LD conditions. These flies carry a null allele of *Pdfr* ($Pdfr^{han5304}$) [1] and a strong hypomorphic allele of *cry* [2–4]. We reasoned that in the absence of the cell-autonomous CRY photoreceptor, entrainment would be more dependent on network interactions. Wild-type (WT), $Pdfr^{han5304}$, $Pdfr^{han5304}$;cry^{b}/+, and cry^{b} flies show robust evening anticipation in LD, with *Pdfr* mutants displaying an advanced phase, as previously observed (Figures 1A–1D) [1, 5]). In contrast, $Pdfr^{han5304}$;cry^{b} double-mutant flies do not exhibit evening anticipation (Figure 1E).

We also examined whether the presence or absence of PDF neurons had a similar effect in *cry* mutants. To selectively ablate PDF neurons, we expressed the proapoptotic gene *head involution defective* (*hid*) under the control of a GAL4-driven upstream activating sequence (UAS*hid*). *hid* expression was driven by GAL4 under the control of the *Pdf* promoter (*Pdf*GAL4). Previous studies have shown consistent ablation of PDF neurons with this approach [6, 7]. As observed in *Pdfr* mutants, *Pdf*GAL4/UAS*hid* and *Pdf*GAL4/UAS*hid*;cry^{b}/+ flies exhibit robust and phase-advanced evening anticipation (Figures S1A–S1D, available online). On the other hand, no evening anticipation is observed in *Pdf*GAL4/UAS*hid*;cry^{b} flies (Figure S1E).

Because the lights-on response in LD can mask some of the clock-driven morning behavior, we assessed morning behavior on the first day of constant darkness (DD1) after LD entrainment. Mutations in the genes encoding *Pdf* or *Pdf receptor* (*Pdfr*), or ablation of PDF neurons, result in severely reduced morning behavior but intact evening behavior [1, 5–7]. In addition, a functional clock in the PDF(+) ventral lateral neurons (LNvs) is sufficient for morning behavior [8]. Thus, these PDF(+) cells are often referred to as morning cells (M cells). $Pdfr^{han5304}$;cry^{b}/+ and *Pdf*GAL4/UAS*hid*;cry^{b}/+ flies show very reduced or undetectable morning behavior, similar to that of $Pdfr^{han5304}$ and *Pdf*GAL4/UAS*hid* controls (Figures 1F–1I and S1F–S1I) and consistent with a role for PDF(+) M cells and PDF signaling in driving morning behavior. Surprisingly, in either $Pdfr^{han5304}$;cry^{b} or *Pdf*GAL4/UAS*hid*;cry^{b} flies, we observe a morning activity peak around the time of subjective lights-on but no apparent evening peak (Figure 1J and Figure S1J). These data indicate that *Pdfr* and *cry* double mutants display a profound change in LD behavior, with a loss of evening behavior but a gain of morning behavior.

A Subset of Evening Cells in *Pdfr* and *cry* Double Mutants Show Molecular Oscillations That Are Antiphase to Those in WT Flies

The lack of evening anticipation in $Pdfr^{han5304}$;cry^{b} flies indicates that either the core molecular clock or the output of clocks important for evening behavior is disrupted. To assay core-clock phase and amplitude, we examined molecular oscillations of the core-clock component, PERIOD (PER), in the interconnected network of ~150 circadian pacemaker neurons [9, 10], including specific clusters important for driving morning and evening behavior. Ablation of cells that do not express PDF—the PDF(−) small ventral lateral neurons (s-LNvs), dorsal lateral neurons (LNds), and subsets of dorsal circadian neurons (DN1s and DN3s)—results in a loss of evening behavior but a retention of morning behavior (in contrast to M cell ablation), and these cells are thus designated evening cells (E cells) [7].

Here, we performed PER immunolabeling in $Pdfr^{han5304}$;cry^{b} and cry^{b} flies at four time points in LD. Consistent with published observations, cry^{b} flies display robust oscillations in several groups of circadian pacemaker neurons (Figure 2). These include the PDF-expressing s-LNvs important for robust morning behavior, as well as E cells, including the single PDF(−) s-LNv, LNds, and a subset of dorsal circadian neurons (DN1) [7, 11]. Of note, we find that the LNds exhibit reduced amplitude oscillations relative to WT (data not shown) [2, 11]. In addition, the other subset of PDF-expressing pacemaker neurons, the large ventral lateral neurons (l-LNvs), do not show any significant oscillation in cry^{b} flies as previously

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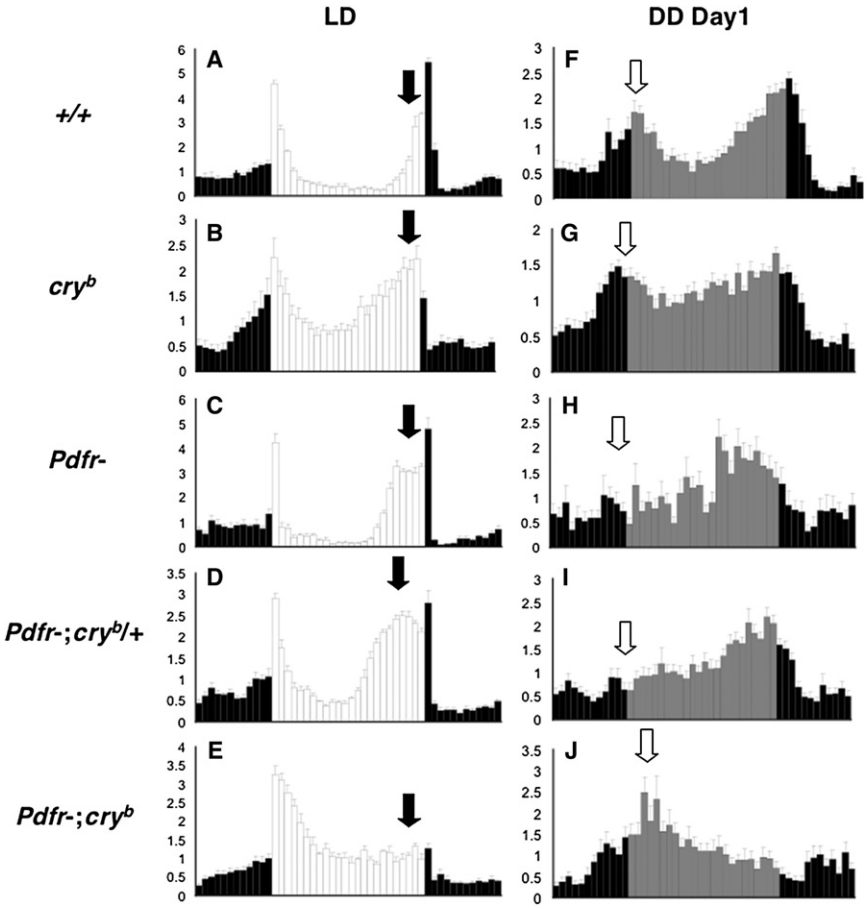

Figure 1. Flies Deficient for PDFR and CRY Exhibit No Evening Behavior and Robust Morning Behavior

(A–E) Normalized activity plots for adult male populations, averaged over four days of 12L:12D entrainment. Light phase is indicated by white bars, and dark phase is indicated by black bars.

(F–J) Normalized activity plots of adult male populations over the last 6 hr of LD (ZT18–CT0) followed by the first 18 hr of DD (CT0-18). Subjective light phase (CT0–CT12) is indicated by dark gray bars, and subjective dark phase is indicated by black bars.

Fly genotypes are as follows: (A and F) +/+; (B and G) cry^b; (C and H) $Pdfr^{han5304}$; (D and I) $Pdfr^{han5304};cry^b/+$; (E and J) $Pdfr^{han5304};cry^b$. Error bars represent standard error of the mean (n = 30–45). White arrows indicate morning behavior, and black arrows indicate evening behavior. Abbreviations are as follows: CT, circadian time; ZT, Zeitgeber time.

reported [2, 11]. $Pdfr^{han5304}$ mutants display robust oscillations with reduced peak PER levels in both the l-LNvs and LNds [12]. Although there are differences between *Pdfr* and *cry* mutants in some groups of circadian neurons (e.g., l-LNvs, another subset of dorsal neuron [DN2], and perhaps the LNd) [11, 13], both mutants display intact rhythms with a phase similar to the WT in the s-LNv, LNd, and DN1 (Figure 2) [12].

In $Pdfr^{han5304};cry^b$ flies, we find that PER oscillations are strongly affected in subsets of pacemaker neurons. In a subset of E cells, the LNds, and the PDF(−) s-LNv (the latter is identified by its lack of PDF immunolabeling), PER oscillations are approximately 12 hr out of phase relative to the cry^b control and *Pdfr* mutants (Figures 2A and 2C) [12]. Considering that $Pdfr^{han5304}$ flies do not exhibit morning anticipation, the most likely explanation for the robust morning anticipation observed in $Pdfr^{han5304};cry^b$ flies is that this behavior is actually driven by the antiphase LNd and PDF(−) s-LNv clocks. Given that we observe similar morning behavior in *Pdf*GAL4/UAS*hid*;cry^b flies, it virtually excludes the possibility that the PDF neurons are driving this behavior.

In addition, PER oscillations are largely abolished in the DN1s of $Pdfr^{han5304};cry^b$ flies, whereas PER oscillations in these cells are intact in $Pdfr^{han5304}$ and cry^b single mutants (Figures 2B and 2C) [12]. To address whether the DN1 cluster might be desynchronized, we examined the standard deviation (SD) of PER intensities within DN1s of a given brain. If DN1s within a single brain are cycling but desynchronized, then one would expect to observe high SDs in PER levels within a brain compared to a brain in which DN1 PER oscillations or levels are synchronous. However, we did not observe a larger within-cluster SD among the DN1s of $Pdfr^{han5304};cry^b$ in comparison to cry^b flies (Table S1). Therefore, we believe that lack of PER oscillation in the DN1s is probably the result of a damped PER oscillation at the single-cell level in the DN1 group, rather than desynchrony among individual DN1s. In addition, we observe constitutive trough levels, not the intermediate levels that would be expected for purely desynchronized oscillators (Figures 2B and 2C). These results demonstrate that the net effect of PDFR signaling is to increase PER levels in the DN1s. Lack of both PDFR and CRY appears to have a modest effect on the amplitude of PER oscillations in the s-LNvs as well (Figures 2A and 2C). Taken together, these data suggest that in the absence of CRY, PDFR plays an important role in setting the phase of [PDF(−) s-LNv and LNd], sustaining (DN1), or amplifying (s-LNv) the oscillation of the molecular clock.

Disruption of Entrained Behavior in *Pdfr* and *cry* Double Mutants Is Specific to Light Entrainment

Our data indicate an important role for PDF signaling in photic entrainment. We hypothesized that PDF signaling may affect nonphotic-entrainment pathways or affect core clocks in ways that would be evident with the use of nonphotic signals. To address this issue, we tested the behavior of $Pdfr^{han5304};cry^b$ flies, as well as $Pdfr^{han5304}$ and cry^b flies, under temperature-entrainment conditions. Flies exhibit anticipatory behavior under temperature cycles, reflecting clock entrainment [14–16]. With the use of 12 hr/12 hr 29°C/21°C thermophase/cryophase (TC) temperature cycles in DD, a locomotor-activity peak anticipating warm-to-cool transition in WT flies is evident (Figure S2A). A previous study has demonstrated that E cell clocks are sufficient for this evening activity peak under temperature cycles [16]. Unlike that which is observed in light entrainment, we do not observe any substantial difference in the phase of evening behavior among $Pdfr^{han5304};cry^b$, $Pdfr^{han5304}$, cry^b, and WT flies entrained to temperature (Figures S2A–S2F). Thus lack of PDF and/or CRY signaling

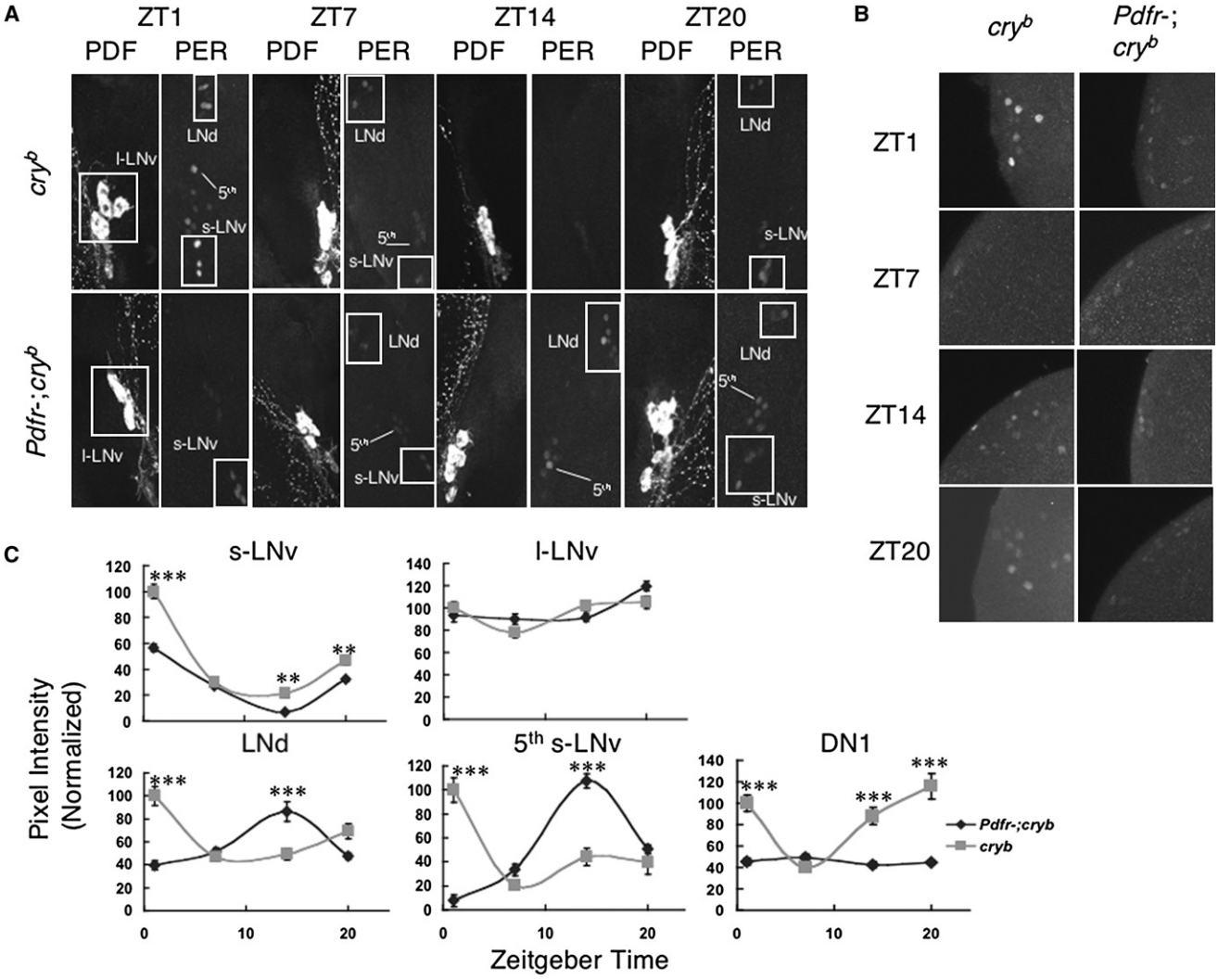

Figure 2. PERIOD Cycles Antiphase in E Cell Subsets of *Pdfr* and *cry* Double-Mutant Flies

(A) Maximum projections of confocal sections taken in representative adult $Pdfr^{han5304}$;cry^{b} and cry^{b} brains labeled with PER and PDF antibodies. Sections contain the LNs at ZT1, ZT7, ZT14, and ZT20. The l-LNvs, PDF(+) s-LNvs, and LNds are in boxes, and the PDF(−) fifth s-LNv is indicated by a line.

(B) Maximum projections of confocal sections taken in representative adult $Pdfr^{han5304}$;cry^{b} and cry^{b} brains labeled with PER antibody. Sections contain the DN1s at ZT1, ZT7, ZT14, and ZT20.

(C) Plots of average normalized pixel intensity versus Zeitgeber time for each pacemaker cell group. See Experimental Procedures for details of quantification method. Error bars represent standard error of the mean. The results are a combination of two independent experiments: s-LNv, n = 40–64; l-LNv, n = 43–77; LNd, n = 41–80; fifth s-LNv, n = 11–17; DN1, n = 50–143. Asterisks mark significant differences among genotypes (two-way ANOVA; single asterisk indicates $p < 0.05$, double asterisk indicates $p < 0.01$, triple asterisk indicates $p < 0.001$).

Abbreviations are as follows: CT, circadian time; ZT, Zeitgeber time.

does not appear to alter circadian locomotor behavior during temperature entrainment. This suggests that PDFR is required specifically for light-mediated entrainment of the evening oscillator.

Potent Disruption of Entrained Behavior in *Pdfr* and *cry* Double Mutants Is Not Evident in Flies Lacking *Pdfr* and the Visual System

The visual system plays an important role in entrainment to LD cycles, especially in the absence of CRY [11]. We observe that *GMR-hid*/+;cry^{b} flies, in which the visual system (i.e., the compound eyes, ocelli, and H-B eyelet) is ablated by *hid* expression [17], entrain poorly to LD cycles, with wide interindividual variation in behavioral phase (Figures S3A–S3F). When these variant individuals are averaged together, little or no rhythm is evident in *GMR-hid*/+;cry^{b} flies (Figures S3G–S3J). This is consistent with a previous study [17] and suggests that pacemaker neurons, including evening oscillators, are entrained to LD cycles mainly via CRY- and visual-system-mediated pathways.

Given the role of the visual system in entrainment to LD cycles, we asked whether loss of the visual system in *Pdfr* mutants also resulted in disruption in entrained behavior comparable to that of *Pdfr* and *cry* double mutants. We examined circadian locomotor behavior of $Pdfr^{han5304}$;*GMR-hid*/+ flies. In LD, $Pdfr^{han5304}$;*GMR-hid*/+ flies exhibit robust evening anticipation that is phase advanced, comparable to that of $Pdfr^{han5304}$ flies but dissimilar from the behavior of $Pdfr^{han5304}$;cry^{b} (Figure 1E and Figure S4). Subtle differences among $Pdfr^{han5304}$;*GMR-hid*/+, $Pdfr^{han5304}$, and *GMR-hid*/+ flies

Table 1. Expression of *CK2β-RNAi* in PDF Neurons Delays the Phase of Evening Behavior in *cry* Mutants under 12L:12D Conditions

Genotype	Time of Evening Behavior Offset (Zeitgeber Time)	DD Period (Hr)	DD Power	DD Rhythmic (%)	N
+/+	14.0 ± 0.0	23.9 ± 0.1	82 ± 8	97	30
*Pdf*GAL4-UAS*CK2βRNAi*/+	13.9 ± 0.1	30.6 ± 0.2	23 ± 2	57	89
cry^b	13.7 ± 0.2	24.2 ± 0.1	53 ± 8	76	32
*Pdf*GAL4-UAS*CK2βRNAi*/+;cry^b	16.2 ± 0.3	27.2 ± 0.4	19 ± 6	50	21

suggest that PDF and the visual system may have functions independent of each other. These results demonstrate that when only the CRY pathway is present, lack of PDF signaling ($Pdfr^{han5304}$;*GMR-hid*/+) does not lead to severely altered behavior, whereas on the other hand, when only the visual pathway is present, lack of PDF signaling ($Pdfr^{han5304}$;cry^b) results in antiphase evening behavior. Given the important role of both the visual system and PDF in CRY-independent entrainment, it is possible that PDF participates in visual system entrainment in cry^b mutants.

PDF Neurons More Strongly Reset Evening Behavior in the Absence of CRY

Prior studies had shown that the pacemaker network switches control between M and E cells in response to different photoperiods [18]. In short photoperiods (10 hr light: 14 hr dark; as in winter), M cells largely set the the timing of morning and evening locomotor behavior. On the other hand, in long photoperiods (as in summer), E cells set the timing of morning and evening behavior. We refer to this light- or photoperiod-dependent switch in the identity of the master pacemaker as M-E network hierarchy. It has been suggested that light activates the output of E cells and inhibits the output of M cells [19]. However, the identity of the molecular mechanisms underlying M-E network hierarchy remains to be elucidated. Given the dramatic alteration of phase and amplitude in E cells (LNds, PDF(−) s-LNv, and DN1s) of flies lacking both PDFR and CRY, we hypothesized that CRY may balance PDF signaling in determining M-E network hierarchy. If true, then PDF(+) M cells would more strongly reset E cell clocks in *cry* mutants than they would those in WT flies.

To address this question, we tested WT and cry^b flies expressing RNAi targeting the regulatory beta subunit of CK2 (*CK2betaRNAi*) under a standard 12 hr light:12 hr dark (12L:12D) condition [5]. CK2 is a protein kinase critical to the timing of the *Drosophila* circadian clocks [20, 21]. Expression of *CK2betaRNAi* in PDF(+) M cells in WT background results in long periods in DD (Table 1), similar to reported partial loss-of-function alleles of *CK2beta* [21]. Nonetheless, in LD, altering the M cell clock does not significantly alter the timing of evening behavior (Figures 3A and 3B), suggesting that manipulating M cell clocks does not alter E cell clocks in 12L:12D conditions, similar to reported results under 12L:12D and 14L:10D [18]. However, when expressing *CK2betaRNAi* in cry^b background, we now observe a significant phase delay of evening behavior in LD ($p < 0.001$), in particular, in the offset of evening behavior relative to cry^b and *Pdf*GAL4-UAS*CK2beta RNAi*/+ flies (Figures 3B–3D, Table 1). We find that on DD1,

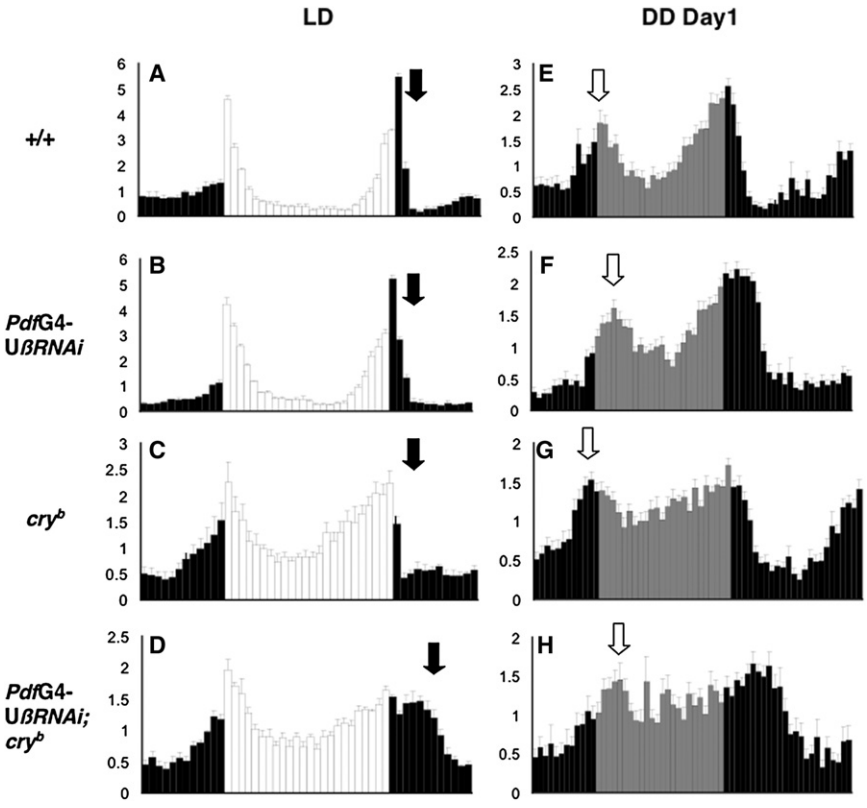

Figure 3. *cry* Mutant Flies Expressing *CK2beta RNAi* in PDF Neurons Exhibit Phase-Delayed Morning and Evening Behavior under Standard LD Entrainment Conditions of 12L:12D

(A–D) Normalized activity plots for adult male populations, averaged over 4 days of 12L:12D entrainment. Light phase is indicated by white bars, and dark phase is indicated by black bars. (E–H) Normalized activity plots of adult male populations over the last 6 hr of LD (ZT18–CT0) followed by the first 24 hr of DD (CT0–CT24). Subjective light phase (CT0–CT12) is indicated by dark gray bars, and subjective dark phase is indicated by black bars.

Fly genotypes are as follows: (A and E) +/+; (B and F) *Pdf*GAL4-UAS*CK2betaRNAi*/+; (C and G) cry^b; (D and H) *Pdf*GAL4-UAS*CK2betaRNAi*/+; cry^b. Error bars represent standard error of the mean (n = 20–89). White arrows indicate morning behavior, and black arrows indicate evening behavior. Abbreviations are as follows: CT, circadian time; ZT, Zeitgeber time.

the phase of morning behavior is delayed in both *Pdf*GAL4-UAS*CK2betaRNAi*/+ and *Pdf*GAL4-UAS*CK2betaRNAi*/+;*cry*^{b} flies relative to WT and *cry*^{b} flies, consistent with the predicted effects of *CK2betaRNAi* on M cell clocks (Figures 3E–3H). The delay in the evening peak cannot be attributed to differences in circadian period, because *Pdf*GAL4-UAS*CK2betaRNAi*/+ flies actually have a longer period than do *Pdf*GAL4-UAS*CK2beta RNAi*/+;*cry*^{b} flies (Table 1). These results demonstrate that the ability of M cells to reset the phase of evening behavior under 12L:12D conditions is evident only in the absence of *cry*.

Discussion

Here, we demonstrate an unexpectedly large role for PDFR signaling in synchronization (or entrainment) of circadian pacemaker neurons to LD cycles. Flies mutant for both *Pdfr* and *cry*, in contrast to single mutants for either gene, display radically altered LD behavior and molecular oscillations. We find that the PDF(+) M cells set the phase of evening behavior in LD in *cry* mutants but not in WT flies, indicating a CRY-dependent change in oscillator dominance. We hypothesize that CRY may gate PDF regulation of E cell phase, providing a framework for photoperiod-dependent changes in oscillator dominance.

It is intriguing that some E cells exhibit antiphase activity and molecular rhythms in the absence of both CRY and PDF signaling. Antiphase behavior is evident under light, but not temperature, entrainment. As in flies, both visual and nonvisual photoreceptors participate in circadian entrainment in mammals [22]. Mice with only a residual amount of rod function exhibit a switch from nocturnal to diurnal activity rhythms accompanied by reversal of clock gene expression in the suprachiasmatic nucleus (SCN) [23]. It is believed that this switch of temporal niche is a result of light entrainment via rod pathways, which is known for its role in night (dim light) vision [23]. We propose that in the absence of PDF and CRY signaling, the visual system uses novel mechanisms to entrain the LNds and the PDF(–) s-LNv to a novel phase.

The reciprocal influence of PDF(+) M cells and non-PDF E cells on each other, as well as morning and evening behavior, depends on photoperiod [18]. On the basis of our results, lack of CRY mimics the effect of less light (or short photoperiod) on the hierarchy of the pacemaker network. Changing the pace of M cells changes the phases of both morning and evening behavior in *cry* mutants, whereas in WT flies, changing the pace of M cells changes only the phase of morning behavior, the latter being consistent with published literature [18]. It has been observed that the visual system suppresses M cell output that drives constant-light condition (LL) locomotor rhythms [19]. Our data together with these data suggest the presence of two distinct pathways operating in LL or long days: an output pathway that is suppressed by the visual system and an M-to-E circuit that is regulated by CRY. In fact, PDF neurons have been shown, in multiple cases, to influence the period of E-cell-driven LL locomotor rhythms [18, 19, 24], indicating that an M-to-E circuit is intact in LL. Our data suggest that CRY directly or indirectly regulates this pathway.

To summarize, we demonstrate that PDF signaling and CRY collaborate to regulate the phase and amplitude of E cell clock in LD. PDF signaling may be modulating visual-system-mediated light entrainment of E cell clock. Moreover, lack of CRY enhances the ability of M cells to phase-reset evening behavior. These findings provide a framework for understanding how photoperiod may determine oscillator dominance in the circadian network. We propose that in long days, light-activated CRY function renders E cell clocks relatively insensitive to PDF signaling, whereas in short days, light-activated CRY function is more temporally restricted, thus allowing PDF signaling to affect clock phase. Thus, the balance between these dual pathways, light-mediated PDFR activity from M cells and CRY signaling, may dictate network hierarchy under different photoperiods.

Experimental Procedures

Fly Rearing and Strains

Flies were reared on cornmeal-agar medium at 22°C –25°C. All entrainment for molecular and behavioral testing was conducted at 25°C except where indicated. *y w* or *w* (for temperature-entrainment experiments) are used as WT strains. *w* and *y w* show similar behavior under temperature cycles [16]. *cry*^{b} [2], *Pdf*^{01} [6], and *Pdf*GAL4 [6] lines were obtained from Michael Rosbash and have been maintained in the lab for nearly ten years. *Pdfr*^{han5304} was obtained from Jaesob Kim [1]. UAS*hid* [25] and *GMR-hid* [26] were obtained from Bloomington Stock Center.

Behavioral Experiments and Analyses

Locomotor activity levels of male flies were monitored with Trikinetics Activity Monitors (Waltham, MA, USA) for up to 5 days of 12L:12D conditions (LD) followed by 7 days of constant darkness (DD). For temperature entrainment, male flies were monitored for 7–12 days of 12 hr 29°C:12 hr 21°C in DD and then released into constant temprature of 25°C in DD. For LD analyses (Figure 1, Figure 3, Figure S1, Figure S4), activity levels from each fly were normalized and averaged within genotypes over 4 days, as described previously [27]. For DD1 analyses (Figure 1, Figure 3, Figure S1), activity levels were normalized and averaged over the first day of DD. For LD5 through DD4 analysis (Figure S3), activity levels were normalized and averaged over the last day of LD and the first four days of DD. For temperature-entrainment analyses (Figure S2), activity levels from each fly were normalized and averaged within genotypes over 4 days.

To calculate the time of offset of evening anticipation in LD (Table 1), we determined the largest 1.5 hr decrease in normalized average activity for each fly over the first 6 hr after lights-off. The time designation refers to the end point of the maximal activity decrease, as averaged among individual flies in each genotype.

PDF and PER Immunohistochemistry, Microscopy, and Quantification

Male flies were entrained in LD for at least 5 days at 25°C. Flies were anesthetized with CO_2 and dissected in 3.7% formaldehyde diluted in PBS. After fixing for 30 min at room temperature, the brains were rinsed two times in PBS and incubated in PBS with 1% Triton for 10 min at room temperature. The brains were then incubated with 5%–10% goat or donkey serum diluted in PBT (PBS with 0.3% Triton) for 30 min at room temperature, followed by overnight incubation of 1:500 rat anti-PDF and 1:4000 rabbit anti-PER (both antibodies are generous gifts from M. Rosbash) in PBT containing 5%–10% goat or donkey serum at 4°C. After several PBT rinses, the brains were incubated with 1:500 goat-anti-rat AlexaFluor488 (Amersham) for PDF immunostaining and 1:500 goat-anti-rabbit AlexaFluor 594 (Amersham) for PER immunostaining in PBT overnight at 4°C. Final rinses in PBT and PBS were followed by mounting in 80% glycerol diluted in PBS. All slides were coded for sample identity and remained so until the numerical-analysis stage. PDF- and PER-stained specimens were photographed with a 60× oil lens on a Nikon Eclipse 800 laser scanning confocal microscope. For a given experiment, the microscope, laser, and filter settings were held constant. PER immunostaining was quantified from digitally projected Z stacks with ImageJ (U.S. National Institutes of Health). We outlined PER-stained soma to obtain average pixel intensity. In each projected Z stack, an unstained area was used for background subtraction. All background-subtracted intensity measurements within a condition (time and genotype) were averaged. For display in Figure 2, we adjusted all PER immunostaining images equally for brightness and contrast to more easily visualize cell groups and brain borders. This does not alter the quantification. To combine experiments, we scaled background-subtracted measurements to ZT1 (first hour of Zeitgeber time) of the control genotype in that experiment. Statistical analysis was conducted in STATISTICA and Excel.

To analyze synchrony among DN1s within a brain, we examined PER intensities within a single hemisphere and calculated the standard deviation among individual DN1s for that hemisphere. For a given genotype and time

point, we then calculated the average standard deviation. A higher standard deviation indicates variation among DN1s within a hemisphere, which in turn reflects desynchrony.

Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01.

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